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PROPERTIES OF TISSUE SPECIFIC MACROPHAGES BEFORE AND AFTER *IN VITRO* ACTIVATION

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Macrophages derived from different tissues: bone marrow, spleen, peritoneal cavity and alveolus, were examined from the aspects of their morphology and functional characteristics [expression of Fc receptors (FcR), phagocytic activity towards yeast particles and nonspecific esterase (NSE) content] before and after in vitro activation. Twenty four-hour-adherent cells were isolated with the aim of analyzing the characteristics of resident tissue macrophages. Following cultivation in vitro 8-day-adherent cells were used to investigate the influence of macrophage activation on their morphology and function. Morphological analysis of cell smears, performed in respect to cell size, showed significant enlargement, especially in the population of alveolar cells cultured for 8 days and activated with colony-stimulating factors (CSFs) and lymphokines. It was also demonstrated that 24-hour- and 8-day-adherent macrophages derived from different tissues exhibited similar properties. All these cells were more than 90% FcR-positive (FcR+), NSE-positive (NSE+) and had phagocytic properties. However, within the population of alveolar macrophages there were some NSE+ cells lacking FcR and phagocytic activity, even after in vitro activation. These results confirmed that the properties of alveolar macrophages differing from those of macrophages from other tissues were dependent on their microenvironment.

Key words: Fc receptor expression, nonspecific esterase, macrophage morphology, phagocytosis

INTRODUCTION

Tissue macrophages are the major component of the classical reticuloendothelial system, which is a dynamic cellular system with the potential to exert a modulatory role in inflammatory responses and tissue homeostasis. The term "macrophages" comprises a morphologically and functionally heterogeneous cell population, which differ widely with respect to size, enzyme activity, phagocytic capacity and a variety of surface markers (Walker, 1976; Lee, 1980). Their functional diversity appears to be related to their anatomical site, which indicates adaptation to local microenvironments within the various tissues. Differences in

macrophage biochemistry, morphology and function have been extensively studied in alveolar and peritoneal macrophages but data concerning the characteristics of lymphoid tissue macrophages are rather obscure. Based on the finding that normal "resident" tissue macrophages could be activated by immunological stimuli to raise an immune response, (Snyder *et al.*, 1982; Steeg *et al.*, 1982), we suggested that the tissue specific difference between macrophages could be eliminated in identical culture conditions.

The aim of this study was to evaluate the functional capacity of macrophages from different tissues, and correlate the functional activities to their morphological properties. Cells were obtained from bone marrow, spleen, peritoneal cavity and alveolus. To avoid dendritic cell involvement 24-hour-adherent cells were analyzed (Steinman and Cohn, 1973). The growth and development of isolated cells was maintained by the use of L-cell line 929 conditioned medium as a source of CSFs (Suzu and Motoyoshi, 2002; Akagawa, 2002). The impact of macrophage activation on their morphology and functional capability was analyzed in the presence of Con A-stimulated spleen cell supernatant (Watson *et al.*, 1979). The data obtained in our experimental model demonstrated tissue micro-environment dependence of macrophage morphology and function, because differences between alveolar and other tissue macrophages detected 24 hours after isolation could not be completely annulled in the presence of CSF-1 and lymphokines.

MATERIALS AND METHODS

Animals. 6-8 week old male or female mice (C57BL/6) were obtained from the Academy of Military Sciences, Belgrade.

Cell preparation. Cell suspensions were made from bone marrow, spleen, peritoneal cavity and lungs. Bone marrow (BM) was obtained from the femur by flushing the shaft from the proximal side with 1 ml culture medium. Spleen was isolated aseptically and spleen cells (SC) were obtained by gently mincing the organ through a stainless steel mesh. The end bronchial lavage technique was performed as described by Holt (1979). Lavage fluids were obtained after the injection of 1 ml phosphate buffer saline (PBS) via the trachea. Lung washings from 4-5 individual animals were pooled. Peritoneal cells (PC) were obtained from mice by peritoneal lavage. Peritoneal washings were withdrawn aseptically through a small incision in the lower right abdominal quadrant. After the injection of 5 ml of PBS, the abdomen was kneaded gently and lavage fluid was collected. After isolation from different sources, the cells were centrifuged at 200 \times g for 10 min and suspended in RPMI 1640 medium (Gibco), containing 100 IU/ml of penicillin, 100 μ g/ml of streptomycin, 2 mM L-glutamine and 10% fetal calf serum (FCS, Gibco).

Cell culture. Macrophage enrichment was achieved by a simple-one-step adherence procedure. Cell suspensions were plated into 100 mm plastic Petri dishes and incubated at 37 °C in 5% CO₂ in a humidified atmosphere incubator (Hereaus) for 24 hours (24-hour-adherent cells) or for 8 days (8-day-adherent cells). When the macrophages were isolated after adherence for 8 days, cultures contained 10% (v/v) L-cell line 929-conditioned medium as the source of CSF

(Stewart *et al.*, 1975). After a 96 hour-incubation with CSF, the culture medium was removed and replaced with fresh medium containing 10% supernatant for Con A-activated spleen cells (Anderson and Gronvik 1979) and incubated for another 96 hours. After washing the monolayer with PBS in order to remove non-adherent cells 24-hour- or 8-day-adherent cells were removed from the plastic surface by incubating with PBS containing 0.02% disodium EDTA for 20 min at 4°C, and then squirting the solution on the dishes vigorously. Next, PBS was added and the remaining adherent cells were scraped off with a rubber policeman. Cells were washed twice with PBS and resuspended in RPMI 1640 medium. Cell were counted in a hemocytometer and viability was determined with 0.1% trypan blue.

Detection of Fc receptors. The EA rosette test was used to detect Fc receptors expressed on the macrophages. Equal volumes of 2% sheep red blood cells (SRBC) and inactivated anti-SRBC serum were incubated for 30 min at 37°C, and then the coated red cells were washed twice in the medium. Sensitized SRBC (5×10^7) and putative macrophages (1×10^6) were centrifuged for 5 min at 100xg (Kedar *et al.*, 1974). After incubation for 60 min at 4°C, the number of rosette forming cells was determined. A cell with five or more attached SRBC was considered a rosette; at least 100 rosettes were analyzed in each test.

Phagocytosis. Phagocytic activity was evaluated by incubation of adherent cells with yeast particles in the presence of neutral red. After incubation for 15-20 min at 37°C, phagocytosis was stopped by addition of EDTA. Cells were washed three times and the number of cells containing yeast particles was determined.

Cellular analysis. Cytocentrifuge prepared smears of cell suspensions were used to study the morphology of the adherent cells. The smears were air dried, stained with May Grünvald and Giemsa stain, and analyzed by light microscopy.

Cytochemical staining for nonspecific esterase. Cytocentrifuge prepared smears were fixed in 10% formalin foams, washed and air dried. The smears were stained with pararosaniline in the presence of α -naphthyl acetate as the substrate (Mueller *et al.*, 1975). After incubation for 60 min at room temperature smears were washed and analyzed by light microscopy.

RESULTS

Morphology of 24-hour-adherent cells

Within the suspensions of adherent cells, macrophages were defined according to the following criteria: size and shape of the cells as well as the appearance and size of the nucleus. Nearly all isolated cells, irrespective of their tissue origin, showed macrophage morphology (more than 94.9%) following 24 hour of cultivation *in vitro* (Table 1). However, significant differences in size were observed for cells originating from different sources. Thus, the highest percentage of "small" macrophages occurred within cell populations from bone marrow and spleen, while more "large" cells were present in the suspension of macrophages isolated from the lungs and peritoneal cavity.

Table 1. Macrophage content and cell size analysis of 24-hour-adherent cells

*source of cells	macro-phage	% of cells according to the size (μm)					
		<15	16-20	21-25	26-30	31-35	>36
BMC	95.3	28.3	35.7	23.5	9.7	2.6	0.0
SC	94.9	30.5	37.0	23.9	8.7	0.0	0.0
PC	95.7	1.0	14.0	42.0	29.0	4.0	0.0
AC	98.1	0.0	26.2	54.9	31.2	0.0	0.0

*Adherent cells were isolated from the suspension of bone marrow cells (BMC), spleen cells (SC), peritoneal cells (PC) and alveolar cells (AC).

Morphology of 8-day-adherent cells

Macrophages of different sizes were also found in populations of 8 day cultivated cells and among lymphokine activated macrophages (Table 2). They were generally larger than 24 hour-adherent macrophages. "Large" cells predominated in the population derived from the peritoneal cavity and especially from the alveolus.

Table 2. Macrophage content and cell size analysis of 8-day-adherent cells

*source of cells	macro-phage	% of cells according to the size (μm)					
		<15	16-20	21-25	26-30	31-35	>36
BMC	97.8	5.0	38.0	30.0	18.0	7.0	2.0
SC	95.0	10.4	15.6	20.8	37.7	13.0	1.3
PC	98.9	0.0	9.1	45.5	41.0	4.6	0.0
AC	99.4	0.0	0.0	1.4	11.1	48.6	38.9

*Adherent cells were isolated from the suspension of bone marrow cells (BMC), spleen cells (SC), peritoneal cells (PC) and alveolar cells (AC).

Fc receptor expression, phagocytosis and NSE staining of 24-hour-adherent cells

The functional analysis of adherent cells included the following examinations: Fc receptor expression on membrane, capability of cells to phagocytize yeast particles and presence of NSE in the cytoplasm. More than 90% of the adherent cells isolated from bone marrow, spleen and peritoneal cavity after 24 hour of cultivation expressed Fc receptors (Figure 1). These cells had phagocytic capability towards yeast particles (87.8 to 94.1%) and contained NSE (91.0 to 96.0%). Bone marrow derived rosette-forming cells are shown in Figure 2.

Slightly different results were obtained with adherent cells isolated from lungs. Although almost all alveolar adherent cells were NSE-positive (95.5%), only

some of those cells expressed Fc receptors (57.8%) and phagocytized yeast particles (50.0%).

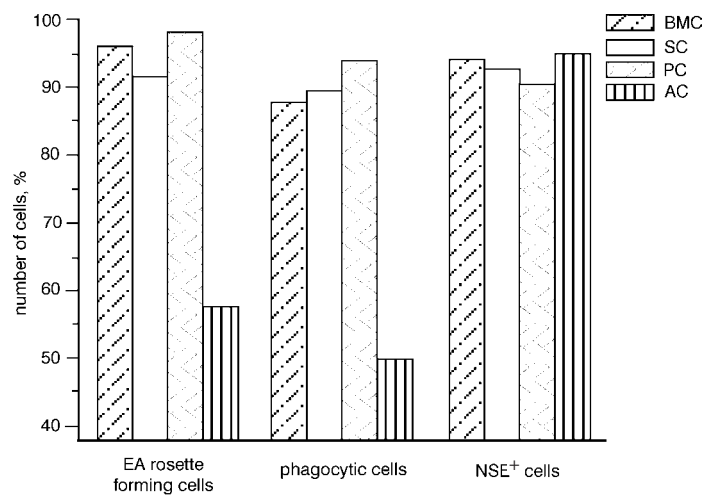


Figure 1. Fc receptor expression, phagocytic and cytochemical characteristics of 24-hour-adherent cells

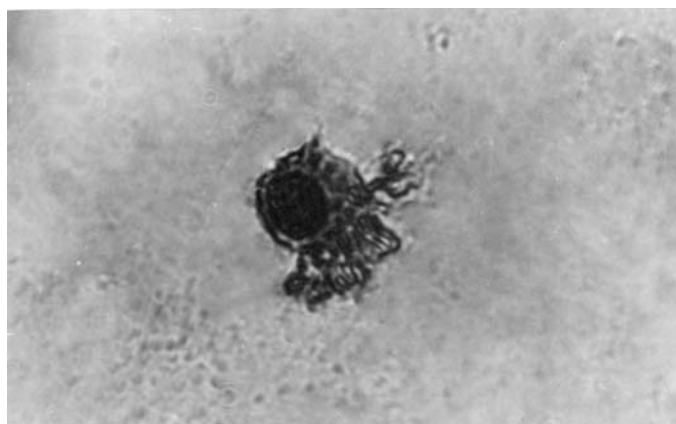


Figure 2. Rosette-forming cells derived from bone marrow (x 1000)

Fc receptor expression, phagocytosis and NSE staining of 8-day-adherent cells

It was found that 95.0% of all cultured peritoneal adherent cells expressed Fc receptors (Figure 3). All these cells showed phagocytic activity and contained NSE. Similar results were obtained with adherent cells isolated from spleen and

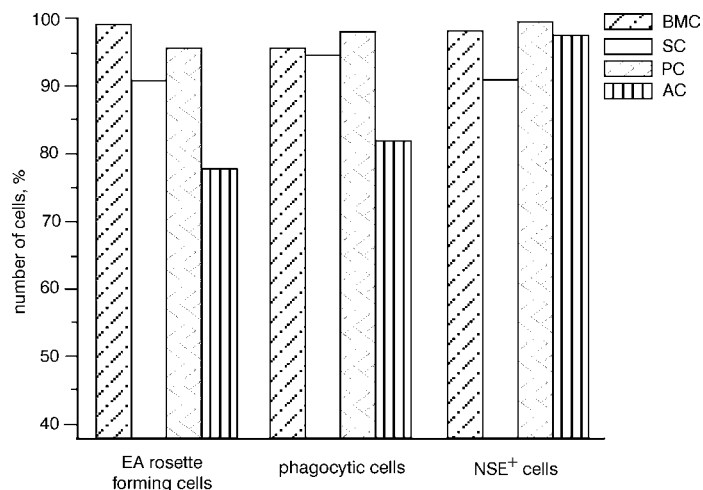


Figure 3. Fc receptor expression, phagocytic and cytochemical characteristics of 8-day adherent cells

bone marrow. There was a positive correlation between the number of FcR-bearing cells, phagocytic activity and the number of NSE⁺ cells. Compared to the results obtained with 24-hour-adherent cells, the *in vitro* culture conditions and lymphokine activation increased the number of FcR⁺ cells within the population of alveolar macrophages. It was clearly demonstrated that all FcR⁺ cells were capable to phagocytize yeast particles (77.5% and 81.6%, respectively). Nevertheless, the number of FcR⁺ and phagocytic cells was still lower than the number of NSE⁺ cells (97.2%).

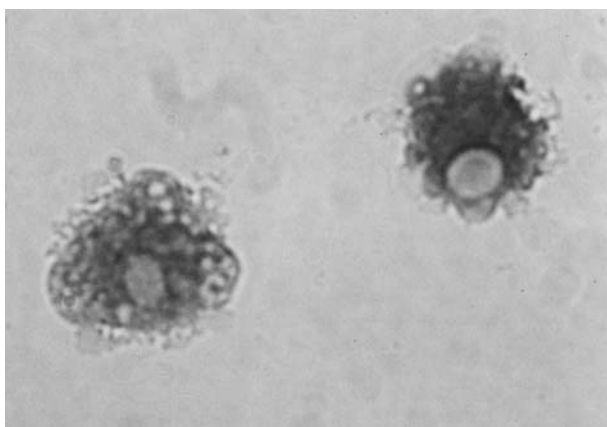


Figure 4. Adherent alveolar macrophages obtained from 8 day-old cell culture, stained for nonspecific esterase (x 1000)

According to our results, NSE content was much higher in alveolar macrophages (Figure 4), than in peritoneal macrophage cell populations (Figure 5) as well as in macrophages from bone marrow and spleen.

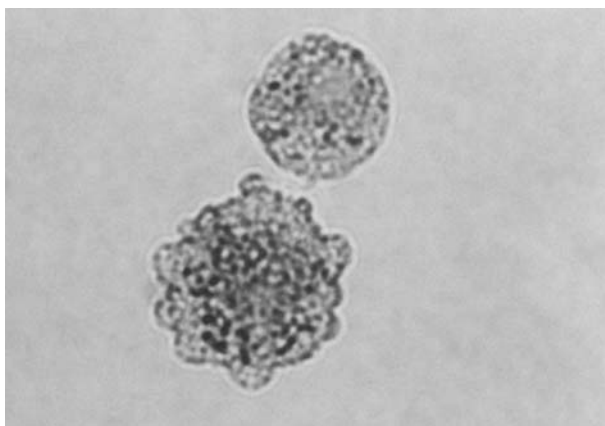


Figure 5. Adherent peritoneal macrophages obtained from 8 day-old cell culture, stained for nonspecific esterase (x 1000)

DISCUSSION

In this study we analyzed 24-hour-adherent cells to define the subpopulations of resident tissue macrophages isolated from different sources, together with 8-day-adherent cells, activated *in vitro* by the use of CSFs and lymphokines, because inflammatory and immunological stimuli could activate macrophages and change their characteristics (Snyder *et al.*, 1982; Steeg *et al.* 1982). There are reports that macrophages, presumably of a relatively immature phenotype, can undergo DNA synthesis at the sites of inflammation in response to CSF-1 (Bitterman *et al.* 1985; Jutila and Banks, 1986). Although adherence to a plastic surface could change the characteristics of resident macrophages to a certain degree, cultivation in the absence of CSFs and lymphokines was used in order to classify these cells as a "resident". Macrophages isolated from the 8-day-old culture in the presence of CSF and lymphokine-containing medium were considered as "activated".

It has already been shown that macrophages in various tissues during steady state as well as at sites of inflammation are heterogeneous with respect to phenotype and function (Chan *et al.* 1998). Light microscopic examination of 24-hour-adherent macrophages showed that they were smaller and rounder when compared with 8-day cultured macrophages, which were larger and more irregular in shape cultivated in macrophage-(M) CSF and lymphokine containing medium. In our culture conditions, peritoneal, and especially alveolar macrophages, enlarged more than bone marrow and spleen macrophages. Moreover, 8-day-

adherent cells from the lungs contained "large" cells mainly (87.5% larger than 30 μm).

Adherent cells isolated from bone marrow, spleen and peritoneal cavity expressed consistent properties. Thus, 24-hour-adherent cells isolated from different sources, as well as 8-day-adherent cells, contained more than 90% of NSE+ cells. Although almost all adherent cells from bone marrow, spleen and peritoneal cavity were NSE+, alveolar adherent cells had the highest amount of NSE in the cytoplasm. These findings are consistent with other data regarding the lysosomal content of macrophages and indicated that the enzyme content of alveolar macrophages was higher than that of peritoneal macrophages (Cohn and Wiener, 1963).

Beside changes in cell size revealed by comparison of resident macrophages and activated macrophages, changes in FcR expression and phagocytic ability were also detected. A high percentage of cells with macrophage morphology isolated from bone marrow, spleen and peritoneal cavity expressed receptors for Fc and could phagocytize yeast particles. The culture conditions did not significantly influence FcR expression or phagocytic activity. Surprisingly, half the alveolar macrophages did not form rosettes with antibody-coated SRBC and some cells were FcR-, even after *in vitro* activation. This could be due to the lack of the membrane receptor for the Fc fragment of immunoglobulins or low avidity of the receptor for the appropriate antibody. It has been shown (Rhodes, 1975) that peritoneal and alveolar macrophages differ in binding of antibodies via membrane FcR and that the mean avidity of the peritoneal macrophage population was approximately three times greater than that of the alveolar macrophages. A 6-fold increase in the proportion of high avidity cells and increase in FcR expression was also reported for normal peritoneal and alveolar macrophages (Rhodes, 1975). Our results are not in full accordance with these findings, as our culture conditions enhanced the number of FcR+ cells, but some (22.5%) alveolar macrophages remained FcR-. Inability to detect FcR on alveolar macrophages could be explained by the fact that the class of receptors on these cells differs from the receptor expressed on other investigated macrophages. It was earlier shown that there are two general classes of Fc γ Rs: activation receptors (e.g., Fc γ RIA, Fc γ RIIIA) and inhibitory receptors (e.g., Fc γ RIIB), which are both present and functional in human and murine macrophages (Ravetch and Bolland, 2001). Activating Fc γ Rs initiates a complex intracellular signaling cascade culminating in enhanced phagocytosis and secretion of inflammatory cytokines (Ravetch and Bolland, 2001). Inhibitory Fc γ Rs, which are activated concomitantly, regulate the threshold of activation responses and ultimately terminate IgG-mediated effector stimulation (Hunter and Indik, 1998). Loor and Roelants (1974) suggested that "small" and "medium" size macrophages contained surface FcR in culture, while "large" macrophages lost these molecules, which is in accordance with the size changes of alveolar macrophages in our experiment. A high percentage of "large" cells in cultured alveolar macrophages could be the reason for the lower number of EA rosettes. It is well known that alveolar macrophages are a heterogeneous cell population and, in response to different stimuli, they mature and become functionally uniform with increased FcR avidity for cytophilyc antibodies (Arend and Mannik, 1973). The re-

sults obtained in this study were not completely in accordance with this statement, since we demonstrated that *in vitro* stimulation of alveolar macrophages increased their ability to form EA rosettes. Nevertheless, all cultured macrophages did not acquire EA rosette-forming activity. The number of FcR+ alveolar macrophages was correlated with the number phagocytic cells in this cell population. Only a 50% of 24-hour-adherent cells were able to phagocytize yeast particles. It was earlier demonstrated that phagocytic ability of alveolar macrophages was less than that of peritoneal macrophages (Walker, 1976). These *in vitro* results are consistent with the results obtained *in vivo* (Poelma *et al.*, 2002) that 70% of alveolar macrophages internalize fluorescent-labeled liposomes with a surfactant-like composition. Previous studies showed that the FcR-gamma deficient mice exhibit multiple defects in FcR-mediated effector cell responses (Takai, 1996), including absence of phagocytic activity against opsonized red blood cells by activated macrophages (Berclaz *et al.* 2002). In our experimental model, 8-days cultivated alveolar macrophages were less capable to phagocytize yeast particles and some of these cells were not phagocytic at all. As the alveolar macrophages are resident professional phagocytic cells, which provide a first line of host defense against microbial pathogens encountered on the respiratory surface, an inability of alveolar macrophages to phagocytize yeast particles is rather unexpected. Although the receptor for CSF is expressed in monocyte/macrophages and their progenitor cells and stimulates both the growth and development of the macrophage (Suzu and Motoyoshi, 2002) it was shown that monocytes cultured *in vitro* in the presence of M-CSF or granulocyte-macrophage (GM)-CSF exhibited different morphology, cell surface antigen expression, and functions (Akagawa, 2002). Bearing in mind the difference between M-CSF-induced macrophages and granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced macrophages *in vitro* (Akagawa, *et al.*, 1988) and the necessity of GM-CSF for growth and differentiation of alveolar macrophages (Lemaire *et al.* 1996; Akagawa *et al.*, 1988), the use of the L-929 conditioned medium as the source of M-CSF could be the reason for the impaired function of alveolar macrophages obtained in this study.

These data are consistent with previous studies on tissue macrophage heterogeneity and provide additional evidence for the concept that macrophages acquire unique characteristics in response to their immune microenvironment. Our experimental model favors the expression of characteristics of primarily resident macrophages as tissue differences persisted even after eight days culture *in vitro*.

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KARAKTERISTIKE TKIVNO SPECIFIČNIH MAKROFAGA PRE I POSLE AKTIVACIJE IN VITRO

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SADRŽAJ

Morfološke i funkcionalne karakteristike makrofaga izolovanih iz različitih organa: kostne srži, slezine, peritonealne šupljine i alveola ispitivane su pre i posle aktivacije u *in vitro* uslovima. Da bi se ispitala kakarakteristike makrofaga koji se nalaze u ispitivanim tkivima analizirane su adherentne ćelije dobijene nakon inkubacije od 24 časa. Uticaj aktivacije makrofaga na njihovu morfologiju i funkciju (ekspresiju Fc receptora, sposobnost fagocitoze čestica kvasca i sadržaj enzima nespecifične esteraze) ispitivan je nakon kultivisanja adherentnih ćelija u toku 8 dana. Morfološkom analizom utvrđeno je značajno povećanje veličine alveolarnih makrofaga kultivisanih tokom 8 dana u prisustvu faktora koji stimulišu rast kolonija i limfokina. Pokazano je da adherentne ćelije iz različitih tkiva izolovane nakon 24 sata i 8 dana imaju slične funkcionalne karakteristike. Više od 90% tih ćelija je ekspresiralo Fc receptore, imalo sposobnost fagocitoze i sadržavalo nespecifičnu esterazu. Međutim, u populaciji alveolarnih makrofaga, pre kao i nakon *in vitro* aktivacije, utvrđeno je prisustvo ćelija koje su sadržavale nespecifičnu esterazu u citoplazmi, ali nisu ekspresirale Fc receptore, niti su imale sposobnost fagocitoze. Ovi rezultati potvrđuju da su karakteristike alveolarnih makrofaga u odnosu na makrofage iz drugih tkiva zavisne od njihovog mikrookruženja.